EXHIBIT B

Exr Helms see indicated section of p601.

Ward, E.S., Gussow, D., Griffiths, A.D., Jones, P.T. & Winter, G. (1989), Binding activities of a repertoir of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature*, 341, 544-546.

Ward, M., Wilson, L.J., Kodama, K.H., Rey, M.W. & Berka, R.M. (1990), Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. *Bio/Technology*, 8, 435-440.

Wu, X.C., Ng, S.C., Near, R.I. & Wong, S.L. (1993), Efficient production of a functional single-chain antidigoxin antibody via an engineered *Bacillus subtilis* expression-secretion system. *Bio/Technnology*, 11, 71-76.

Yu, H., Nakano, Y., Yamashita, Y., Oho, T. & Koga, T. (1997), Effects of antibodies against cell surface protein antigen PAc-glucosyltransferase fusion proteins on glucan synthesis and cell adhesion of *Streptococcus mutans*. *Infect. Immun.*, 65, 2292-2298.

In vitro production of recombinant antibody fragments in *Pichia pastoris*

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Introduction

The methyltropic yeast *Pichia pastoris* is rapidly becoming a preferred host for the efficient expression of heterologous proteins (reviewed in Hollenberg and Gellissen, 1997; Sreekrishna *et al.*, 1997; Sudbery, 1996). *P. pastoris* combines the general features of protein expression in eukaryotes with the fast growth and genetic modifiability of prokaryotes. It is less expensive than other eukaryotic expression systems, such as baculovirus or mammalian tissue culture, and it typically yields higher quantities of secreted functional proteins. The ability to correctly express folded secreted proteins, including highly disulphide-bonded ones (White *et al.*, 1994), provides a distinct advantage over bacterial systems that often require laborious and inefficient procedures to denature and refold proteins expressed as insoluble, inclusion bodies (Skerra, 1993). *P. pastoris* is a superior expression host over its more famous non-methyltropic relative, *Saccharomyces cerevisiae*, because *P. pastoris* grows stably to high cell densities in fermentors (>100 mg dry weight per ml culture) and it has strong, tightly regulated promoters (Ellis *et al.*, 1985). For these reasons, *P. pastoris* has recently been exploited as an expression system for the high-level secretion of many proteins, including recombinant antibody fragments.

By definition, methyltropic yeasts are capable of utilizing methanol as their sole carbon source. The first enzyme in the methanol-utilization pathway, alcohol oxidase (AOX), is encoded by two closely related genes: *AOX1* and *AOX2* (Ellis *et al.*, 1985; Cregg *et al.*, 1989). Although the AOX1 and AOX2 proteins have 97% sequence identity and equivalent enzymatic activity, over 95% of the alcohol oxidase activity in *P. pastoris* is attributable to AOX1. This is due to the strength of the *AOX1* promoter (Cregg *et al.*, 1989; Koutz *et al.*, 1989). Upon the addition of methanol, *AOX1* gene transcription is rapidly induced to high levels and ultimately accounts for 5% of the total polyA$^+$ RNA (Cregg and Madden, 1988). The AOX1 protein is correspondingly overexpressed and comprises up to 30% of the total intracellular protein. Heterologous genes are therefore cloned under control of the *AOX1* promoter to

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allow for their rapid and strong induction by methanol. The expression of heterologous genes driven by the *AOX1* promoter can result in protein yields exceeding 200 mg/l in shake flask cultures, and can be in the g/l range in fermentation cultures (Faber *et al.*, 1995; Cregg *et al.*, 1993).

Generating and screening transformants

To maximize the stability of protein expression, heterologous genes are integrated into the *P. pastoris* genome. A series of expression vectors designed for homologous integration are commercially available (Invitrogen, San Diego, USA; www.invitrogen.com). The salient features of these vectors are the inclusion of the 5' and 3' regions of the *AOX1* gene (to target plasmid integration via homologous recombination), transcription termination and polyadenylation signals, and the *HIS4* gene (for selecting yeast transformants able to grow in histidine-deficient media) or the Zeocin resistance gene (for selecting both bacterial and yeast transformants). Some vectors contain additional features such as the f1 origin of replication (for mutagenesis of single-stranded DNA), the kanamycin resistance gene (for selecting G418-resistant high copy number transformants), or yeast-derived signal sequences (for protein secretion). Plasmids containing the gene of interest are linearized and then integrated in the yeast genome via homologous recombination upon transformation by spheroplasting or electroporation. We routinely use electroporation because of its simplicity and relatively high efficiency (10^3-10^4 transformants per μg of DNA).

The usual goal of screening transformants is to identify high protein-expressing "jackpot" clones. Although expression levels are affected by the integration site, gene sequence, and the strain of *P. pastoris* transformed, the most profound effect typically results from copy number (Clare *et al.*, 1991). High copy number integrants are identified genetically by PCR (Linder *et al.*, 1996; Haaning *et al.*, 1997) or immunologically with specific antibodies (McGrew *et al.*, 1997; Wung and Gascoigne, 1996). Screening procedures such as these can be combined with genetic or selection strategies designed to enrich for high copy integrants. For example, plasmids with multicopy inserts can be generated prior to transformation, or high copy number integrants can be selected for their increased resistance to the drug G418 if the integrated plasmid contains the kanamycin resistance gene (Scorer *et al.*, 1994).

Secreted vs. intracellular expression

Induced proteins are expressed intracellularly or are secreted, depending on the absence or presence of an appropriate signal sequence. The utility of exploiting the secretory pathway is that *P. pastoris* can be grown to high cell densities, and then induced with methanol, in inexpensive, chemically defined, protein-poor media. The low level of protein in the media formulations facilitates detection and purification of the final secreted product, which can comprise the vast majority (80-90%) of the total protein in the supernatant (Faber *et al.*, 1995). Secreted proteins are easily and efficiently purified from the supernatants, often in one step by affinity or metal-chelating chromatography (Eldin *et al.*, 1997).

Expression of recombinant antibody fragments

The first recombinant antibody fragment reported to be expressed in *P. pastoris* was a rabbit single chain Fv (scFv) selected from a bacterial phage display library (Ridder *et al.*, 1995). ScFv fragments contain heavy and light chain variable regions connected by a small, flexible peptide (Huston *et al.*, 1988; Bird *et al.*, 1988). These fragments can be modified to increase their stability or avidity (e.g. disulphide-stabilized scFv and bivalent scFv fragments), and to add additional specificities or effector functions (e.g. bispecific diabodies or scFv-immunotoxins) (table I). ScFv fragments are well suited for many *in vivo* diagnostic and therapeutic applications because their reduced size (27,000-30,000 M_r) permits them to penetrate tissues more rapidly than whole antibodies and to be cleared more rapidly from the blood (Yokota *et al.*, 1992). Because the *in vivo* use of scFv fragments and their derivatives often requires large quantities of protein, it is not surprising that most antibody fragments expressed in *P. pastoris* have clinical potential.

The reported levels of scFv production in *P. pastoris* range from 10 to 250 mg/l in shake-flask cultures (table I). These fragments are almost invariably expressed as secreted proteins in minimal media to facilitate their purification. However, Luo *et al.* (1997b) recently reported that a scFv fragment was induced to comparable levels (200 mg/l) in yeast grown in the standard buffered minimal methanol medium or in a completely protein-free medium. The ability to produce large amounts of secreted proteins in essentially phosphate buffer should make large-scale protein preparations in *P. pastoris* even more attractive.

Disadvantages of protein expression in *P. pastoris*

Not every protein can be expressed to high levels in *P. pastoris* due to factors such as codon bias and the requirement for particular post-translational modifications (Sreekrishna *et al.*, 1997). However, shuttle vectors exist that allow the same gene to be expressed in bacterial, yeast, or mammalian cells

Table I. Antibody fragments secreted by *P. pastoris.*

Ab fragment	Antigen specificity	Yield	Reference
scFv	Leukaemia inhibitory factor	> 100 mg/l	(Ridder *et al.*, 1995)
scFv	Squamous carcinoma	10-50 mg/l	(Luo *et al.*, 1995; Luo *et al.*, 1996)
dsFv		NR(*)	
scFv-chelator		NR	
dsFv-chelator		NR	
scFv	Thomsen-Friedenreich (pan-adenocarcinoma)	200 mg/l	(Luo *et al.*, 1997b)
bivalent scFv	CA125-ovarian carcinoma	100 mg/l	(Luo *et al.*, 1997a)
bivalent diabody	Carcinoembryonic antigen	1 mg/l	(FitzGerald *et al.*, 1997)
bispecific diabody	CEA/CD3	1 mg/l	
scFv	Desipramine	250 mg/l	(Eldin *et al.*, 1997)
scFv	CD7	60 mg/l	
scFv/B7-2	erbB2/CD28	0.5 mg/l	(Gerstmayer *et al.*, 1997)

(*) Not reported.

(White *et al.*, 1994, 1995; Liu *et al.*, 1998). These vectors permit investigators to choose the expression system that best fits their particular need, be it high-yield or appropriate post-translation modification, with minimal genetic manipulations. The size of the protein to be expressed may also be limiting because to our knowledge, there are no reports of proteins greater than 117 kDa (β-galactosidase) being expressed in *P. pastoris*. High yield protein production in *P. pastoris* is dependent on several factors such as adequate aeration, methanol concentration and temperature maintenance at 28°-30°C (*P. pastoris* is not thermotolerant). All of these conditions have to be maintained to ensure optimal inductions but recent advances in batch-fed fermentation (White *et al.*, 1995; Chiruvolu *et al.*, 1997; Jimenez *et al.*, 1997; Chen *et al.*, 1997) and shake-flask technologies (Guarna *et al.*, 1997) should facilitate monitoring induction conditions.

Conclusions

We have found *P. pastoris* to be an inexpensive and robust system for the high-level production of antibody fragments. To find that "jackpot" clone, we recommend the following strategy: clone the relevant gene into a plasmid that contains a signal sequence, the kanamycin resistance gene, and an epitope- or histidine-tag sequence, select for transformants with increased G418 resistance, and then screen colony lifts of induced transformants with tag-specific antibodies. An alternate strategy is to linearize the plasmid separately with different restriction endonucleases to target integration at various sites in the *Pichia* genome. Differentially linearized vectors could be mixed in the same transformation to generate multiple integrants per clone. This increases the likelihood that the gene of interest integrates in a transcriptionally active site dependent on local chromatin structure.

A recent report of alpha-sarcin ribotoxin production in *P. pastoris* suggests that this system may soon be exploited for the expression of scFv immunotoxins (Martinezruiz *et al.*, 1998). Larger, intact antibodies such as camelid heavy chain IgG (Hamers-Casterman *et al.*, 1993) might also be expressed, since *P. pastoris* can secrete functional disulphide-bonded homodimers and heterodimers (Luo *et al.*, 1997a; FitzGerald *et al.*, 1997; Kalandadze *et al.*, 1996). We therefore anticipate that the use of *P. pastoris* as an expression system for antibody-derived fragments will be on the rise.

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References

Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.-M., Lee, T., Pope, H.S., Riordan, G.S. & Whitlow, M. (1988), Single-chain antigen-binding proteins. *Science*, 242, 1038-1041.

Chen, Y.L., Cino, J., Hart, G., Freedman, D., White, C. & Komives, E.A. (1997), High protein expression in fermentation of recombinant *Pichia pastoris* by a fed-batch process. *Process Biochem.*, 32, 107-111.

Chiruvolu, V., Cregg, J.M. & Meagher, M.M. (1997), Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fedbatch fermentations. *Enzyme Microb. Technol.*, 21, 277-283.

Clare, J.J., Rayment, R.B., Ballantine, S.P., Sreekrishna, K. & Romanos, M.A. (1991), High level expression of tetanus toxoid fragment C in *Pichia pastoris* strains containing multiple tandem integrants of the gene. *Bio/Tech.*, 9, 455-460.

Cregg, J.M. & Madden, K.R. (1988), Development of the methyltropic yeast, *Pichia pastoris*, as a host system for the production of foreign proteins. *Dev. Ind. Microbiol.*, 29, 33-41.

Cregg, J.M., Madden, K.R., Barringer, K.J., Thill, G.P. & Stillman, C.A. (1989), Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*. *Mol. Cell. Biol.*, 9, 1316-1323.

Cregg, J.M., Vedvick, T.S. & Raschke, W.C. (1993), Recent advances in the expression of foreign genes in *Pichia pastoris*. *Bio/Tech.*, 11, 905-910.

Eldin, P., Pauza, M.E., Hieda, Y., Lin, G.F., Murtaugh, M.P., Pentel, P.R. & Pennell, C.A., (1997), High-level secretion of two antibody single chain Fv fragments by *Pichia pastoris*. *J. Immunol. Methods*, 201, 67-75.

Ellis, S.B., Brust, P.F., Koutz, P.J., Waters, A.F., Harpold, M.M. & Gingeras, T.R. (1985), Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast, *Pichia pastoris*. *Mol. Cell. Biol.*, 5, 1111-1121.

Faber, K.N., Harder, W., Ab, G. & Veenhuis, M. (1995), Methylotrophic yeasts as factories for the production of foreign proteins. *Yeast*, 11, 1331-1344.

FitzGerald, K., Holliger, P. & Winter, G. (1997), Improved tumour targeting by disulphide stabilized diabodies expressed in *Pichia pastoris*. *Protein Eng.*, 10, 1221-1225.

Gerstmayer, B., Altenschmidt, U., Hoffmann, M. & Wels, W. (1997), Costimulation of T cell proliferation by a chimeric B7-2 antibody fusion protein specifically targeted to cells expressing the erbB2 proto-oncogene. *J. Immunol.*, 158, 4584-4590.

Guarna, M.M., Lesnicki, G.J., Tam, B.M., Robinson, J., Radziminski, C.Z., Boraston, A., Jervis, E., Macgillivray, R.T.A. & Turner, R.F.B. (1997), On-line monitoring and control of methanol concentration in shake-flask cultures of *Pichia pastoris*. *Biotechnol. Bioeng.*, 56, 279-286.

Haaning, J., Oxvig, C., Overgaard, M.T. & Sottrupjensen, L. (1997), Simple and reliable procedure for PCR amplification of genomic DNA from yeast cells using short sequencing primers. *Biochem. Mol. Biol. Int.*, 42, 169-172.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Bendahman, N. & Hamers, R. (1993), Naturally occurring antibodies devoid of light chains. *Nature*, 363, 446-448.

Hollenberg, C.P. & Gellissen, G. (1997), Production of recombinant proteins by methylotrophic yeasts. *Curr. Opin. Biotechnol.*, 8, 554-560.

Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. & Oppermann, H. (1988), Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci.* USA, 85, 5879-5883.

Jimenez, E.R., Sanchez, K., Roca, H. & Delgado, J.M. (1997), Different methanol feeding strategies to recombinant *Pichia pastoris* cultures producing high level of dextranase. *Biotechnol. Tech.*, 11, 461-466.

Kalandadze, A., Galleno, M., Foncerrada, L., Strominger, J.L. & Wucherpfennig, K.W. (1996), Expression of recombinant HLA-DR2 molecules. Replacement of the hydrophobic transmembrane region by a leucine zipper dimerization motif allows the assembly and secretion of soluble DR alpha beta heterodimers. *J. Biol. Chem.*, 271, 20156-20162.

Koutz, P.J., Davis, G.R., Stillman, C.A., Barringer, K.J., Cregg, J.M. & Thill, G.P. (1989), Structural comparison of the *Pichia pastoris* alcohol oxidase genes. *Yeast*, 6, 167-177.

Linder, S., Schliwa, M. & Kube-Granderath, E. (1996), Direct PCR screening of *Pichia pastoris* clones. *Bio/Tech.*, 20, 980-982.

Liu, Z., Cashion, L.M. & Pu, H.F. (1998), Protein expression both in mammalina cell lines and in yeast *Pichia pastoris* using a single expression plasmid. *Bio/Tech.*, 24, 266-272.

Luo, D., Mah, N., Krantz, M., Wilde, K., Wishart, D., Zhang, Y. & Jacobs, F. (1995), Vl-linker-Vh orientation-dependent expression of single chain Fv-containing an engineered disulfide-stabilized bond in the framework regions. *J. Biochem.*, 118, 825-831.

Luo, D., Mah, N., Wishart, D., Zhang, Y., Jacobs, F. & Martin, L. (1996), Construction and expression of bifunctional proteins of single-chain Fv with effector domains. *J. Biochem.*, 120, 229-232.

Luo, D., Geng, M., Noujaim, A.A. & Madiyalakan, R. (1997a), An engineered bivalent single-chain antibody fragment that increases antigen binding activity. *J. Biochem.*, 121, 831-834.

Luo, D., Mah, N., Krantz, M., Wishart, D., Jacobs, F. & Martin, L. (1997b), High level secretion of single-chain antibody in *Pichia* expression system. *Biotechnol. Tech.*, 11, 759-761.

Martinezruiz, A., Delpozo, A.M., Lacadena, J., Mancheno, J.M., Onaderra, M. & Gavilanes, J.G. (1998), Secretion of recombinant pro- and mature fungal alpha-sarcin ribotoxin by the methyltropic yeast *Pichia pastoris* - the lys-arg motif is required for maturation. *Protein Expr. Purif.*, 12, 315-322.

McGrew, J.T., Leiske, D., Dell, B., Klinke, R., Krasts, D., Wee, S.F., Abbott, N. & Harrington, K. (1997), Expression of trimeric CD40 ligand in *Pichia pastoris*: use of a rapid method to detect high-level expressing transformants. *Gene*, 187, 193-200.

Ridder, R., Schmitz, R., Legay, F. & Gram, H. (1995), Generation of rabbit monoclonal antibody frag-

ments from a combinatorial phage display library and their production in the yeast. *Bio-Technol.*, 13, 255-260.

Scorer, C.A., Clare, J.J., McCombie, W.R., Romanos, M.A. & Sreekrishna, K. (1994), Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Bio-Technol.*, 12, 181-184.

Skerra, A. (1993), Bacterial expression of immunoglobulin fragments. *Curr. Opin. Immunol.*, 5, 256-262.

Sreekrishna, K., Brankamp, R.G., Kropp, K.E., Blankenship, D.T., Tsay, J.T., Smith, P.L., Wierschke, J.D., Subramaniam, A. & Birkenberger, L.A. (1997), Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene*, 190, 55-62.

Sudbery, P.E. (1996), The expression of recombinant proteins in yeasts. *Curr. Opin. Biotechnol.*, 7, 517-524.

White, C.E., Kempi, N.M. & Komives, E.A. (1994), Expression of highly disulfide-bonded proteins in *Pichia pastoris*. *Structure*, 2, 1003-1005.

White, C.E., Hunter, M.J., Meininger, D.P., White, L.R. & Komives, E.A. (1995), Large-scale expression, purification and characterization of small fragments of thrombomodulin - the roles of the 6th-domain and of methionine-388. *Protein Eng.*, 8, 1177-1187.

Wung, J.L. & Gascoigne, N.R.J. (1996), Antibody screening for secreted proteins expressed in *Pichia pastoris*. *BioTech*, 21, 808-811.

Yokota, T., Milenic, D.E., Whitlow, M. & Schlom, J. (1992), Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.*, 52, 3402-3408.

Production of antibodies in transgenic plants

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A) Plant bioreactors

The first transgenic plants were reported in 1983 (Fraley *et al.*, 1983; Zambryskyi *et al.*, 1983). Since then, many recombinant proteins have been expressed in several important agronomic species of plants including tobacco, corn, tomato, potato, banana, alfalfa (Austin *et al.*, 1994) and canola (summarized in Kusnadi *et al.*, 1997a). Recent work suggests that plants will be a facile and economic bioreactor for large-scale production of industrial and pharmaceutical recombinant proteins (Kusnadi *et al.*, 1997b; Austin *et al.*, 1994; Krebbers *et al.*, 1992; Whitelam *et al.*, 1993). Genetically engineered (transgenic) plants have several advantages as sources of proteins compared with human or animal fluids/tissues, recombinant microbes, transfected animal cell lines or transgenic animals. These include:

(1) production of raw material on an agricultural scale at low cost;

(2) efficiency of the transformation technology and speed of scale-up;

(3) correct assembly of multimeric antibodies (unlike bacteria);

(4) increased safety, as plants do not serve as hosts for human pathogens, such as HIV, prions, hepatitis viruses, etc.;

(5) reduced capitalization costs relative to fermentation methods.

Perhaps most important are the cost benefits of plant production. For example, Kusnadi *et al.* (1997b) calculated the cost of producing a recombinant protein in various agricultural crops (see fig. 1). The cost estimate was based on the commodity price of the crop, the fraction of total protein in the crop, and the not unreasonable assumption that the recombinant protein accumulated to 10% of the total plant protein. Although crops with more protein content (e.g. soybeans, 40% versus potatoes 2%) are more

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